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ACCEPTANCE

This thesis, OXIDIZED SOYBEAN OIL ALTERS THE EXPRESSION OF PPAR GAMMA AND TARGET GENES IN 3T3-L1 CELLS, by Nicole Dingels

was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by committee members in partial fulfillment of the requirements for the degree Master of Science in the College of Health and Human Sciences, Georgia State University.

The Master's Thesis Advisory Committee, as representatives of the faculty, certifies that this thesis has met all standards of excellence and scholarship as determined by the faculty.

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ABSTRACT

Oxidized soybean oil alters the expression of PPAR γ and target genes within 3T3-L1 cells

By Nicole Dingels

Background: The typical western diet contains foods with modest amounts of lipid oxidation products. Previous work by us and others have demonstrated that mildly oxidized lipids promote a gain in fat mass while highly oxidized lipids decrease fat mass in rodents and triglyceride (TAG) accumulation in 3T3-L1 cells. Adipocyte differentiation is regulated by a key nuclear transcription factor known as PPAR γ .

Objective: To investigate if the alterations in triglyceride accumulation in 3T3-L1 cells pretreated with oxidized soy oil are due to 1) a change in PPAR γ DNA interactions 2) changes in the expression of SREBP-1c, PPAR γ , and/or its target genes.

Main Methods: Confluent 3T3-L1 cells were pretreated for 24hours with 0.01% soy oil (SO) which was either unheated (unheated SO) or heated for 3, (3h-SO), 6 (6h-SO), or 9hours (9h-SO). The effect of 24hour soy oil exposure was assessed at several time points throughout the differentiation process. Alterations in PPAR γ DNA interaction was assessed using a PPAR γ transcription factor assay kit while alterations in the expression of genes upstream and downstream of PPAR γ was determined by RT-PCR. Primary and secondary products of oxidation within the SO were determined by spectrophotometry.

Results: The 6hr-SO contained the greatest concentration of peroxides whereas both the 6hr-SO and 9hr-SO contained a significantly higher concentration of conjugated dienes and aldehydes. Nuclear extracts from 3T3-L1 cells pretreated with 6h-SO demonstrated the greatest reduction in PPAR γ DNA binding. Compared to the unheated SO and mildly oxidized 3h-SO, cells treated with the 6h-SO had a significant reduction in SREBP-1c, PPAR γ , LPL, and GLUT4 expression occurring early in the differentiation process. Variations in the gene expression of 6hr-SO pretreated cells persisted within partially differentiated and mature adipocytes.

Conclusions: Pre-treatment of preadipocytes with soy oil heated for ≥ 6 h greatly decreases the activity of PPAR γ in the nucleus and adipogenic gene expression. These changes seen in early differentiation seem to correlate the best with the phenotype of reduced triglyceride accumulation seen in mature adipocytes.

**Oxidized soybean oil alters the expression of PPAR gamma and
target genes within 3T3-L1 cells**

By Nicole Dingels

A thesis

Presented in Partial Fulfillment of Requirements for the
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ABBREVIATIONS

CD: conjugated diene

CT: conjugated triene

C/EBP α : CAAT/enhancer binding protein alpha

LPL: lipoprotein lipase

PCR: polymerase chain reaction

PPAR γ : peroxisome proliferator activated receptor gamma

PUFA: polyunsaturated fatty acid

PV: peroxide value

SO: soybean oil

SREBP-1c: sterol regulatory enhancer binding protein-1c

TAG: triglyceride

TZDs: thiazolidinediones

3h-SO: soybean oil heated for 3-hours

6h-SO: soybean oil heated for 6-hours

9h-SO: soybean oil heated for 9-hours

9-HODE/13-HODE: 9- or 13-hydroxy linoleic acid

9-HPODE/13-HPODE: 9- or 13-hydroperoxy linoleic acid

Chapter I: Introduction

a. Polyunsaturated fatty acids and the generation of oxidized lipids: The role of food lipids in disease and obesity has been gaining attention as obesity and its related comorbidities continue to rise. The typical Western diet contains high-fat foods, which are heavily fried, heated, and/or processed. These foods generate cytotoxic and genotoxic compounds due to the highly susceptible nature of polyunsaturated fatty acids (PUFAs) to undergo oxidation and structural degradation¹. The oxidation of dietary PUFAs generates primary products of oxidation known as lipid hydroperoxides (e.g 9-HPODE and 13-HPODE) which have a peroxy group attached at the 9th or 13th carbon. These products are highly labile and are quickly converted to hydroxy fatty acids (9-HODE and 13-HODE). Both primary products are known as conjugated dienes (CDs) due to a shift in the position of the double bonds which are now separated by one carbon atom². Extensive oxidation occurring by prolonged heat exposure leads to self-propelling oxidative reactions that result in the breakdown of primary products into secondary products of oxidation known as carbonyls, aldehydes, and conjugated trienes. In the case of omega-6 fatty acids, peroxidation results primarily in the formation of aldehydes known as 4-hydroxyneonenal and hexanal³. A correlation between potentially harmful lipid byproducts, such as 13-HODE, and the diseases of obesity has been reported; however the molecular mechanisms and interactions are unclear^{1,4}. Low-density lipoproteins (LDL) receptor-/- mice fed a high-fat diet supplemented with 13-HODE had a significantly greater aortic lesion area compared to controls consuming a high-fat diet only. Moreover, LDL-/- mice fed a low-

fat diet consisting of oxidized linoleic acid, as 13-HODE, demonstrated a decrease in weight gain but an increase in fat pad mass compared to mice consuming unheated diet⁵.

b. PPAR γ and adipocyte differentiation:

The peroxisome proliferator-activated receptors (PPARs) include a family of nuclear-hormone receptors that are responsible for regulating several metabolic pathways by influencing expression⁶. PPAR- α , PPAR- β/δ , and PPAR γ , the three PPAR isoforms, exhibit tissue-specific distributions with distinct roles in metabolism⁷. Among the various biological functions of the PPAR isoforms, PPAR γ is of interest due to its indispensable role in adipocyte differentiation, triglyceride accumulation (TAG), insulin sensitivity, and the inflammatory response. A number of genes are specific to adipocytes and lipid metabolism: adiponectin, perilipin⁸, 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1)⁹, adipocyte fatty acid binding protein, phosphoenolpyruvate carboxykinase, acyl-CoA synthase, fatty acid transport protein, lipoprotein lipase (LPL), adipocyte P2 (aP2), and GLUT-4. The transcriptional regulation of these genes is contingent on PPAR γ activity and expression,^{6,10} and transcriptional activation of PPAR γ is regulated by both ligand-dependent and independent mechanisms. With dependent mechanisms, ligand binding is followed by the dimerization of PPAR γ with retinoid X receptor (RXR) in the nucleus. The heterodimer then binds to its specific DNA sequences, or peroxisome proliferator response element (PPRE), which triggers the recruitment and binding of coactivators. The activated complex responds by promoting the transcription/repression of specific target genes⁶. Without ligand binding, PPAR γ is inactive; nuclear corepressors bind to the heterodimer and downregulate gene transcription by histone deacetylase recruitment⁸. PPARs are unique transcription factors; they are able to recognize and bind

with a number of diverse ligands including endogenous, exogenous (e.g. dietary), and synthetic/pharmaceutical molecules¹¹.

Oxidized lipids and PPAR γ : Ligand binding is the determining factor behind the involvement of PPAR γ in adipocyte differentiation as the ligand is responsible for both promoting or suppressing PPAR γ activity and its target genes. Interestingly, the primary biological ligand for PPAR γ has yet to be identified and considerable variability exists in the ability of endogenous ligands to activate PPAR γ and influence gene transcription¹⁰. Of the natural ligands, PUFAs and their derivatives, fatty acid metabolites, hormones, and certain prostanoids, have all demonstrated a greater ability to activate PPAR γ compared to inherent fatty acids, which elicit a relatively weak response in terms of PPAR γ activation¹⁰. For example, oleic acid, erucic acid, and linoleic acid were unable to activate PPAR γ in their free, intact form; however the arachidonic acid metabolite, 15-deoxy-*D*12,14-prostaglandin J2, induced activation significantly indicating that fatty acid metabolites and derivatives may be significant and noteworthy biological ligands¹². Examining a variety of fatty acids for potential interactions Nagy *et al.* found oxidized fatty acids to function as potent PPAR γ ligands. In addition, two oxidized metabolites of linoleic acid, 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), were found to be highly effective activators of PPAR γ compared to the unoxidized fatty acids- oleic acid, erucic acid, and linoleic acid¹². Thus, it appears that PPAR γ responds to a variety of ligands that can be obtained endogenously or exogenously from dietary fatty acids. Moreover, it is possible that the effects of 13-HODE in decreasing weight gain in LDL receptor -/- mice may be a result of the interaction between PPAR γ and oxidized lipids. On the other hand, other studies have

demonstrated that dietary oxidized lipids, consumed as heated oils, decrease fat mass but promote glucose intolerance¹³. These differential effects of dietary oxidized lipids might be due to differences in the amount and the level to which lipids are oxidized. Nonetheless dietary oxidized lipids alter adipose mass; it is important to determine if these changes increase or decrease metabolic risk.

Considering the importance of PPAR γ in adipose tissue homeostasis and the influence of diet and nutritional quality on weight gain and obesity, it is plausible that certain dietary components act as PPAR γ agonists/antagonists that modify lipid metabolism. The exploration of exogenous PPAR γ ligand interactions, particularly dietary constituents, may provide meaningful information regarding the unfavorable alterations in lipid metabolism that correlate with obesity. Duque-Guimarães *et al.* provided male adult rats a diet with partially hydrogenated fats, or rich in trans-fatty acids and poor in PUFAs¹⁴. A down-regulation in the mRNA expression of PPAR γ and adiponectin was observed and accompanied by an increased expression in tumor necrosis factor-alpha (TNF- α) and resistin. Rats consuming a fish oil diet, rich in omega-3 fatty acids, demonstrated an increase in adiponectin expression and secretion by way of an increase in PPAR γ expression, while a soy oil diet, rich in omega-6 fatty acids, resulted in a similar yet more modest effect¹⁴. Our lab previously demonstrated a noteworthy interaction between TAG accumulation within 3T3-L1 cells and exposure to varying degrees of oxidized soy oil rich in omega-6 fatty acids. Interestingly, 3T3-L1 cells pretreated for 24 hours with highly oxidized soy oil before the induction of differentiation, demonstrated an inhibition in preadipocyte differentiation based on a significant reduction in TAG accumulation at the end of the differentiation process. The

differentiation of 3T3-L1 cells exposed to unheated and mildly oxidized soy oil was unaltered. Oxidized lipids are of interest considering their high consumption in the typical Western diet¹. Altered PPAR γ activity is apparent in atherosclerosis and studies have shown the proficiency of oxidized low-density lipoproteins (LDL), rich in primary oxidation products 9-HODE and 13-HODE, to activate PPAR γ leading to an increase in macrophage proliferation and foam cell formation¹². Considering the high intake of fried oils and foods in the United States, the growing rates of obesity and insulin resistance, and the connection between oxidized LDL and PPAR γ in atherosclerosis, it is presumable that oxidized dietary PUFAs alter genetic expression through modulation of PPAR γ . The alterations in TAG accumulation, weight gain, and glucose homeostasis in the presence of highly oxidized lipids may be a result of an interaction with PPAR γ . If correct, these findings would have profound effects on our understanding of the development of obesity and diabetes and may ultimately influence preventive and therapeutic strategies. In addition, these findings may provide a better understanding of PPAR γ ligand interactions.

Objectives: The objective of this study is to investigate if alterations in triglyceride accumulation in 3T3-L1 cells mediated by oxidized soy oil are due to 1) a change in the ability of PPAR γ to bind to its DNA response element and/or 2) changes in the expression of PPAR γ , its target genes LPL, GLUT-4, and upstream SREBP-1C.

Chapter II: Literature Review

1. Molecular regulation of adipocyte differentiation:

Preadipocyte differentiation into triglyceride-storing mature adipocytes is essential for the development of adipose tissue, a metabolically active organ necessary for normal growth and metabolism¹⁵. Differentiation is directly affected by PPAR γ ligand interactions and indirectly by transcription factors, which have complementary binding sequences on the promoter region of PPAR γ thereby influencing the expression and

activity of PPAR γ ⁷ (Figure 1). Examining the expression of transcription factors aids in identifying the underlying mechanism(s) behind alterations in adipocyte differentiation associated with exposure to a specified ligand.

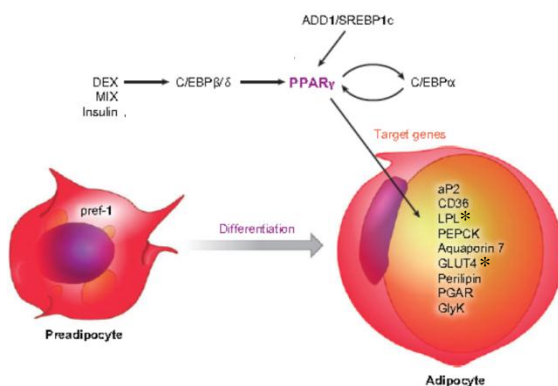


Figure 1: PPAR γ pathways of gene transcription, regulation, and adipogenesis; Modified figure from Spiegelmen and Tontonto¹⁰. Target genes of interest are denoted by a *.

a. Upstream factors that regulate PPAR γ expression:

CAAT/enhancer binding proteins (C/EBP) are a family of helix loop transcription factors that are necessary for PPAR γ expression and activity at the cellular level. Within preadipocytes, C/EBP- β and - δ induce PPAR γ by binding to its promoter sequence; the presence of a bound ligand is required for the fabrication of PPAR γ target genes as well as C/EBP α . The relationship between C/EBP α and PPAR γ is highly

dependent on PPAR γ as C/EBP α alone cannot compensate the adipogenic actions within PPAR γ knockout models. By self-regulation, C/EBP α directly maintains PPAR γ expression; it also appears to be necessary for the physiological actions of PPAR γ and adipose homeostasis. The presence of insulin resistance within embryonic fibroblasts derived from C/EBP α deficient mice has been observed^{16,17}.

Sterol regulatory element binding protein, also known as adipocyte differentiation and determination factor-1 (**SREBP-1c**), is a transcription factor from the leucine zipper family that affects PPAR γ activity during the early stages of adipocyte differentiation. SREBP-1c prompts the gene expression of several adipogenic enzymes involved in fatty acid synthesis⁷ and may also incite the synthesis of endogenous ligands that promote PPAR γ transcriptional activity^{7,17}. Moreover hepatic SREBP-1c binds dietary PUFAs, but not saturated or monounsaturated fatty acids, resulting in a decrease in SREBP-1c activity, a decrease in mRNA expression, and the suppression of lipogenic genes¹⁸. Inhibition of SREBP-1c interferes with adipogenesis by blocking adipocyte differentiation and PPAR γ expression¹⁶. While the physiological mechanisms remain elusive, studies have shown PPAR γ ligands to alter the expression and protein levels of PPAR γ and those of C/EBPs and SREBP-1c¹⁰⁻¹³. Ezure *et al.* observed an increase in PPAR γ and C/EBP α , but not C/EBP- β or - δ , within 3T3-L1 preadipocytes exposed to a dietary agonist¹⁹. Exposure to a PPAR γ antagonist is known to inhibit adipogenesis and triglyceride accumulation. Treatment with phosphorylated glycosamine suppresses PPAR γ , C/EBP α , and SREBP-1 protein levels and mRNA expression within differentiated adipocytes¹⁷.

b. Downstream factors influenced by PPAR γ in Adipocyte Differentiation:

In considering the effects of factors located upstream of PPAR γ , analyzing the outcome of specific target genes located downstream of PPAR γ , including LPL and GLUT-4¹⁰, in the presence of various PPAR γ ligands provides valuable insight pertaining to their individual influence on differentiation, TAG accumulation, and their ability to act as a ligand and promote or inhibit PPAR γ activity.

LPL and GLUT-4 are absolutely essential to TAG accumulation and their expression is regulated by PPAR γ . **LPL** is necessary for the hydrolysis of TAGs, allowing free fatty acids to enter the adipocyte for reesterification and lipid accumulation²⁰. **GLUT-4** transports glucose into the cell; glucose functions as the glycerol backbone source for TAG synthesis²¹. The influence of various PPAR γ ligands, including both agonists and antagonists, on gene expression and markers of differentiation has been explored extensively within murine 3T3-L1 preadipocytes and human models. Of note, 3T3-L1 cells are a well-recognized preadipocyte murine cell line that provides an appropriate model for adipogenesis. These cells are fully capable of differentiation, and express the target genes of interest, which includes GLUT-4²². Differentiation within this cell line has been shown to require 15-16 days for full adipocyte maturation with TAG accumulating capabilities²³. This process is dependent upon cell exposure to an adipogenic cocktail consisting of fetal bovine serum, dexamethasone, isobutylmethylxanthine, and insulin¹⁶. The necessity of PPAR γ for adipocyte maturation has been demonstrated within 3T3-L1 PPAR γ knockout cells based on their inability to differentiate after exposure to several potent agonists, or ligands, along with a reduction in glucose uptake by way of a decrease in GLUT-4 levels²⁴.

Furthermore, Liao *et al.* noted PPAR γ to be unnecessary post-differentiation. Protein expression and TAG accumulation within mature adipocytes was unaffected by PPAR γ deletion²⁴. On the other hand, Tontonoz and Hu *et al.* suggested the necessity of PPAR γ for the perpetuation of aP2 and PEPCK levels in mature, fully differentiated adipocytes²⁰. In association with the increase in PPAR γ expression in the presence of the potent rosiglitazone (RTZ) agonist, Leyvraz *et al.* reported an increase in LPL mRNA expression within a human preadipocyte cell line and within primary preadipocytes derived from obese women⁹. These findings infer the differentiation of preadipocytes to mature triglyceride storing adipocytes based on the presence of LPL that is specific to mature adipocytes. Similarly, in the same experiment GW6992 (2-chloro-5-nitro-N-phenyl-benzamide), a PPAR γ antagonist, prevented LPL and adiponectin expression and these findings were paralleled by an expected decrease in TAG accumulation⁹. Interestingly, Nidhina *et al.* found an increase in the expression of PPAR γ and GLUT-4 within 3T3-L1 cells exposed to vanillin²⁵, demonstrating widespread diversity in potential dietary ligands. The influence of different ligands on adipogenic gene expression is of interest considering the rising rates of obesity, diabetes, and coinciding alterations in lipid metabolism and partitioning.

2. The need for natural ligands of PPAR γ :

Currently, synthetic agonists, such as the thiazolidinediones (TZDs) drugs used to treat diabetes, exhibit a greater affinity for PPAR γ than any other binding ligand³⁻⁵. These PPAR γ agonists are beneficial in the management of diabetes by increasing insulin sensitivity in skeletal muscle and inhibiting hepatic gluconeogenesis. Moreover, TZDs

reduce plasma free fatty acids by PPAR γ activation, which enhances the ability of adipocytes to sequester lipids for storage thereby reducing the effects of lipotoxicity in non-adipose tissues²⁶. The increase in fat mass or adiposity within TZD users is accompanied by unfavorable side effects including hepatic toxicity, edema, and cardiovascular disease risk. TZDs have provided an invaluable source of information pertaining to the physiological mechanisms of PPAR γ ; however their toxic side effects resulted in the removal of troglitazone (Rezulin) from the market. Pioglitazone and rosiglitazone remain on the market with heightened risk of severe health consequences¹¹. The currently active quest for the development of new drugs capable of modulating PPAR γ activity without the adverse side effects encourages and continues to expand our knowledge of the mechanistic foundation from which PPAR γ operates. In addition, dietary components that act as agonists or antagonists are included in this search and may provide a valuable link between diet, genetics, and obesity. The genetic expressions of PPAR γ and several downstream genes are altered in obesity indicating an unspecified modification in PPAR γ activity^{21,27}. A better understanding of PPAR γ ligand interactions is warranted considering the role of PPAR γ in adipogenesis. This insight may elude to a better understanding between the development of obesity and alterations in gene expression.

Chapter III: Methods

a. Oxidation of Soybean oil

Refined soybean oil, USP (85% unsaturated fatty acid profile; LOT# 41-1336), was obtained from Welch, Holme and Clark Co., Inc. Two hundred milliliters of the fresh, unoxidized soybean oil (unheated SO) was aliquoted into small vials and stored under nitrogen at -80°C. The remaining 600ml was oxidized by continuous exposure to oxygen, and was heated at 193-210°C for varying time periods. Oxidation time, or heat exposure, did not start until the oil reached 195°C. Soy oil was heated for a total of nine hours with the removal of 200ml every three hours; soy oil was oxidized for a total of 3, 6, and 9 hours (3hr-SO, 6hr-SO, and 9hr-SO respectively). After heating, the oil was allowed to cool for ten minutes, aliquoted to small vials, and stored under nitrogen at -80°C until further determination of the levels of oxidative products was made.

b. Extraction of Soybean Oil Lipids

Soy oil lipids were extracted using the methods of Radin²⁸. Soy oil was weighed, homogenized in HIP (hexane:isopropanol, 3:2), and transferred to a separatory funnel. After the addition of ten milliliters (ml) Na₂SO₄ to the filtrate, the funnel was shaken vigorously and set aside for twenty minutes allowing for the separation of three distinct phases. The top layer containing the extracted fat was gathered and dried under nitrogen at 40°C using a nitrogen evaporator (N-Evap; Organomation Associates, Inc., Berlin, MA). The extracted oil was weighed, dried under nitrogen and stored at -80°C.

c. Determination of Oxidative Products in Soybean Oil

Soybean oil contains small amounts of lipid hydroperoxides due to the susceptible nature of PUFAs to oxidation; the presence of these oxidation products is significantly increased with heat exposure such as frying²⁹. In particular, dietary oils have been shown to contain varying concentrations of hydroperoxides even before processing³⁰. Lipid oxidation in the unheated soy oil (SO) and oxidized SOs was determined by measuring the presence of both primary and secondary products of oxidation. Primary oxidation products were determined by measuring conjugated lipid peroxides and hydroxides, which contain the conjugated diene (CDs) structure. A shift in the double bond position within the fatty acid forms a CD³¹. As oxidation persists, these primary oxidation products further degrade into secondary products that retain the conjugated double bond: aldehydes, conjugated trienes (CTs), ketones, and epoxy compounds¹.

Primary Products of Oxidation: The PeroxySafe STD kit (SafTest, MP Biomedicals, Solon, OH, USA) was used to determine lipid peroxides in the oil samples. The assay was performed according to kit procedure on unheated SO, 3hr-SO, 6hr-SO, and 9hr-SO in triplicates and read at 570nm and 690nm. A standard calibration curve was performed first and then the four soy oil samples were assayed. Calibration curve with an $R^2 \geq 0.97$ was considered functional. Actual absorbance was obtained by subtracting the absorbance at 690nm from the corresponding 570nm absorbance for each sample. Peroxide concentrations of the individual oil samples were calculated by inserting the actual absorbance value into the equation from the calibration curve. The result was multiplied by its dilution factor allowing the peroxide concentration to be expressed as a peroxide value (PV) in mEq/kg oil. Conjugated dienes were determined by hexane

preparation and dilution. Samples were weighed to obtain equal amounts (~40-44mg) and mixed by vortexing with hexane to make a 1% solution (weight x 100 – volume). Next, soy oil samples were individually diluted with variable amounts of hexane in order to obtain relevant absorbance readings at 234nm in the range of 0.2-0.8 absorbance units. The absorbance of pure hexane was subtracted from the readings at 234nm to obtain the relative units of absorbance and expressed as absorbance at an extinction coefficient at 1% concentration ($E_{234}^{1\%}$). All samples were ran in triplicates and measured using the Epoch spectrophotometer (BioTek, Winooski, VT, USA).

Secondary Products of Oxidation: The presence of aldehydes in the soy oil samples was determined by using the Aldesafe MSA kit (SafTest, MP Biomedicals, Solon, OH, USA). Assay was conducted on all four soy oil conditions in triplicates according to kit procedures with absorbance readings at 550 and 690nm. After running the calibration curve, samples were assayed and read in the spectrophotometer at 550nm and 690nm. The actual absorbance was calculated by subtracting the blank and the 690nm reading from the corresponding 550 absorbance value for each sample. Conjugated trienes are determined using the same methods as for the CDs, except they were read at 268nm and 270nm; therefore all absorbencies were measured alongside CD quantification in order to preserve sample. All samples were ran in triplicates and measured using the Epoch spectrophotometer (BioTek, Winooski, VT, USA).

d. Cell Culture and Soy Oil Exposure

Our current understanding of adipocyte differentiation has been fostered by the use of 3T3-L1 cells, which are able to induce the differentiation of preadipocytes into mature, triglyceride storing adipocytes¹⁷ in the presence of 1-methyl-3-isobutylxanthine

(IBMX), dexamethasone, and insulin¹⁹. The process of 3T3-L1 differentiation completes at approximately day 15 or 16 and has been shown to involve a number of key transcription factors specific to the study of adipogenesis²³.

Murine 3T3-L1 cells (ATCC, Manassas VA) were removed from liquid nitrogen and cultured in a base media of Dulbecco's Modified Eagle medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS), 1% penstrep, and 1% L-Glutamine. T75 and T25 flasks were designated for RNA isolation and protein collection, respectively, while 100mm dishes provided nuclear extracts. Cells were plated and once all flasks/dishes were 100% confluent they were ready for soy oil exposure (**See Figure 2**).

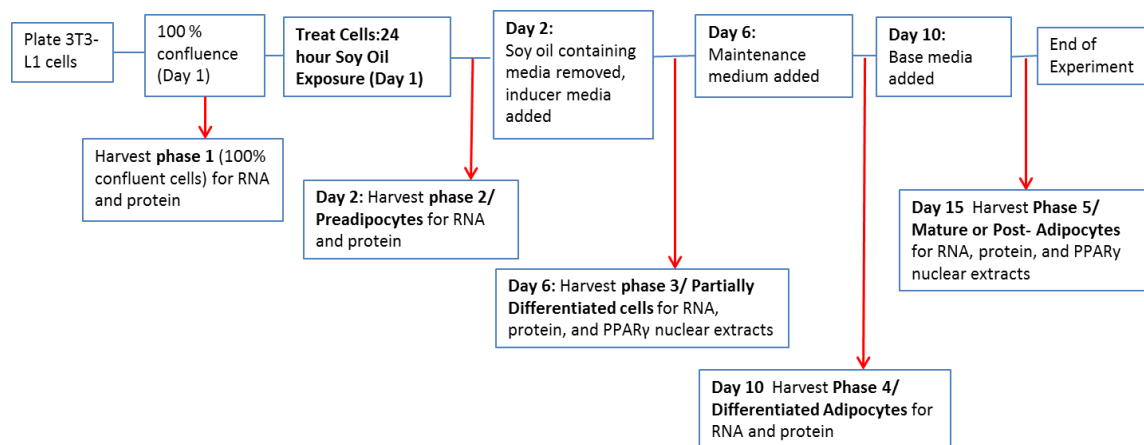
All four soy oil conditions were prepared for cell culture by making 1% soybean oil solutions for each condition using hexane and 0.2% BSA fatty acid free solution. Soy oil was added with hexane to make a 5% concentration. The hexane was evaporated using nitrogen and the soybean oil solution was mixed with 0.2% BSA. The oils were set at room temperature for one hour allowing for the binding of oil to BSA after which they were sterile filtered and stored at -20°C.

On treatment day, each soy oil condition was incorporated at a 0.01% concentration into DMEM with 1% charcoal filtered FBS and 1% pen/strep. The 100% confluent cells were treated with equal amounts of media containing either control, unheated SO, 3hr-SO, 6hr-SO, or 9hr-SO. The control received the same exact charcoal filtered media without any soy oil. On the same day, one T75 flask and one T25 flask containing cells cultured in base media were harvested for RNA and proteins, respectively. Before removal cells were washed in HBSS. T75 flasks were harvested for

RNA isolation using Tri-reagent (Sigma). T25 flasks were harvested for proteins by supplying additional HBSS, followed by scrapping, microfugation, and cell re-suspension in a radio-immunoprecipitation buffer (RIPA, Sigma) with protease inhibitors. The RNA in tri-reagent and the proteins were stored at -20°C until further analysis. After exactly 24-hours of soy oil exposure, cells from one T75 and one T25 flask were harvested for RNA and proteins, respectively, for each of the five conditions: control, unheated SO, 3hr-SO, 6hr-SO, 9hr-SO. RNA and protein were stored at -20°C until further analysis. On the same day, the treatment media was removed from the remaining 30-flasks and 10-dishes; cells were washed with HBSS and provided inducer media consisting of the previously described base media with the addition of 0.1% insulin, 1% 0.5mM IBMX, and 10% 10µM dexamethasone. Four days later, 5 x T75 flasks, 5 x T25 flasks, and 5 x 100mm dishes were removed for RNA, proteins, and nuclear extracts, respectively, of the partially differentiated adipocytes. RNA and proteins were collected as previously described while the nuclear extracts were obtained via a nuclear extraction kit (see section V.). On the same day, the inducer media was removed from the remaining 20-flasks and 5-dishes; cells were washed with HBSS and provided a maintenance media consisting of base media plus 0.1% insulin. Unlike the inducer media, the maintenance media changed color after two days and therefore was changed. Two days later or 4-days after the initial introduction of maintenance media, the differentiated adipocytes from 5 x T75 and 5 x T25 flasks were harvested for RNA and proteins, respectively. The remaining 10 flasks and 5-dishes were continued in base media for five more days allowing the complete maturation of the adipocytes into post-adipocytes. Finally, the RNA, proteins, and nuclear extracts of the fully matured 3T3-L1 cells were obtained as

previously described. A timeline of the 3T3-L1 exposure and cell collections is presented in **Figure 2**.

Figure 2: Timeline of Soy Oil Experiment



e. PPAR γ Nuclear Transcription Activation

3T3-L1 nuclear extracts were isolated according to the Cayman Nuclear Extraction Kit (#10009277). Approximately, $\sim 10^7$ cells were collected within 15ml pre-chilled tubes.

The cells were centrifuged (Eppendorf centrifuge 5810 R) and re-suspended by the addition of specified assay reagents as instructed. The cytosolic and nuclear extracts were stored in pre-chilled vials at -80°C until further analysis. Nuclear protein content for all samples was estimated using the Lowry's Method³². Two and one-half milliliters of Lowry's reagent consisting of 1% CuSO_4 , 2% $\text{NaKC}_4\text{H}_4\text{O}_6$, and 20% Na_2CO_3 was added to 6 μl of sample along with 494 μl distilled H_2O . The mixture was vortexed and set aside for 15 minutes after which 250 μl of 1N Folin-phenol reagent (Sigma) was added. The samples were set aside for 20 minutes and then read in the spectrophotometer at 660nm. All samples were run in duplicates and an average of the two absorbencies was used in

determining protein quantity. The protein concentration per sample amount was determined using the equation from a calibration curve and the curve was generated the same way as the samples with the substitution of bovine serum albumin at five concentration levels. The influence of soy oil exposure on PPAR γ activation was determined using a PPAR γ transcription factor assay kit #10006855 (Cayman, Ann Arbor, MI). The assay was performed according to manufacturer protocol using 10 μ g of each sample. All samples were performed in duplicates and absorbencies were read at 450nm using the Benchmark Plus microplate spectrophotometer (Biorad, Hercules CA).

f. RNA isolation, quality, and quantification

Total RNA was isolated using TRI Reagent (Sigma, Saint Louis, MI). Cells were lysed in 1.5mL of the reagent by repeat pipetting and then centrifuged in the Micro Legend 21R (Thermo Scientific Waltham, MA) at a speed of 12,000g for 10minutes at 4°C. The supernatant was removed and shaken vigorously with 300 μ l of chloroform (VWR, Radnor, PA). Samples were centrifuged at a speed of 12,000g for 10 minutes at 4°C allowing the top aqueous layer to be collected and transferred to clean microfuge tube with 0.750ml isopropanol . After mixing, samples were centrifuged at 12,000g for 10minutes at 4°C. The supernatant was removed leaving the RNA pellet, which was washed in 75% ethanol, centrifuged at 12,000g for 10minutes at 4°C, and then suspended in TE buffer. RNA quality and integrity were determined by gel electrophoresis using 1.2% agarose with TAE and ethidium bromide. One microliter blue/orange loading dye plus 9 μ l RNase free water were added to each sample. A DNA 100kb ladder was used alongside 1 μ l of each sample. The gel was run at 100volts for 50minutes using the Power

Pack 1000 (Biorad, Hercules CA). The presence and identification of RNA was confirmed based on gel electrophoresis using a 100bp ladder. RNA was quantified using a NanoDrop (Thermo Scientific, Wilmington DE) and the Inde 1000 NanoDrop software. RNA purity was confirmed by a 260nm to 280nm absorption ratio between 2.07-2.11nm. RNA samples were diluted with TE to provide 1µg/µl and stored at -20°C until further analysis.

g. Reverse Transcription and real time-polymerase chain reaction (RT-PCR)

Reverse transcription of RNA to cDNA was conducted using the iScript™ cDNA synthesis kit (Biorad, Hercules CA). Following the manufacturer protocol, 1µl RNA (equivalent to 1µg RNA) was mixed with 19µl of the mastermix providing a total volume of 20µl. RNA samples were transcribed using the iCycler (Biorad) with cycling conditions of 5-minutes at 25°C, 30-minutes at 42°C, 5-minutes at 85°C, and an optional holding time at 4°C. Immediately following incubation, samples were stored at -20° overnight. RT-PCR was used to determine the relative expression of genes (Table 1) using iQ™ SYBR® Green Supermix. The reaction mixture consisted of 12.5µl SYBR supermix, 1µl forward primer, 1µl reverse primer, 9.5µl RNase free H₂O, and 1µl cDNA (equivalent to 1µg). All plates were centrifuged at 1000rpm for two minutes and amplified in the iCycler iQ RT-PCR (Biorad, Hercules CA). Cycling condition were 95°C for 3-minutes, followed by 40cycles at 95°C for 10s and 60°C for 20s, 60°C for 10s 7x, and a 4°C holding time. Fluorescence measurement was used continuously to detect gene products. Results generated reflect light cycler/fluorescence threshold values (CTs) calculated via Biorad iQ5 software version 2.0. All samples, including the 18S standard,

were assayed in triplicates. CT results for each gene is expressed in relation to the 18S housekeeping gene and the preadipocytes pretreated with unheated SO. Delta CT (Δ CT) values were generated by subtracting the 18s housekeeping gene from the CT value of the target gene. Next, delta delta CT ($\Delta\Delta$ CT) values were generated by subtracting the CT of the unheated SO exposed cells from the Δ CT value for each target gene. The fold change of each target gene within the soy oil exposed preadipocytes in relation to the unheated SO treatments was calculated by taking 2 and squaring it to the negative $\Delta\Delta$ CT.

Table 1: PCR Primer Sequences

<i>Gene</i>	<i>Primer</i>	<i>Sequences (5'→3')</i>
PPAR γ	F	TGATTTGTCCGTTGTCTTTCC
	R	GTCTCACAATGCCATCAGGTT
LPL	F	ATTGAAACACCTGGCCTTTG
	R	TGAGCCATGTCTTCAACTGC
GLUT4	F	GATTCTGCTGCCCTTCTGTC
	R	ATTGGACGCTCTCTCTCCAA
SREBP-1c	F	ATCGGCGCGGAAGCTGTCGGGGTAGCGTC
	R	ACTGTCTTGGTTGTTGATGAGCTGGAGCT
18S	F	GGCCTCACTAAACCATCCAA
	R	GCAATTATCCCCATGAACG

h. Western Blots

Cellular protein content within 4 μ l of cytosolic fraction for all samples was quantified for Western analysis using the Lowry method previously described. All samples were run in duplicates and an average of the two absorbencies was utilized in determining protein quantity. Various sample volumes (equivalent to 25 μ g) were resolved with 10 μ l of Laemmli buffer and enough dH₂O to provide a total volume of 30 μ l. A 10% gel was prepared using EZ run (Fisher, Fair Lawn NJ), 10% ammonium persulfate, and TEMED. Prior to gel loading the sample-Laemmli mixture was boiled for 5-minutes. The gel was ran at 100 volts for 115-minutes, then was transferred to a nitrocellulose membrane (Whatman, Dassel Germany) at 100 volts for 60-minutes. The blot was confirmed by the presence of bands after the addition of Ponceau S. Primary antibodies: PPAR γ (Rockland, Gilbertsville PA) and actin (Sigma, St. Louis MO). Secondary antibodies: anti-rabbit IgG and anti-actin (Sigma). After completing the PPAR γ western analysis, the blot was stripped according to *Blot Restore* kit (Chemicon, Temecula, CA) and probed with the actin antibody. Band intensity of each blot was detected using the ChemiDoc Image Lab (Biorad, Hercules CA).

i. Statistical Analysis

Data were analyzed with SPSS version 18.0 (IBM Corporation). Data are shown as means \pm SE. Differences in the mean values for the expression of each adipogenic gene of interest between the unheated SO, 3hr-SO, 6hr-SO, and 9hr-SO cells were compared by one-way analysis of variance (ANOVA) for normally distributed data or by Kruskal-Wallis test for non-parametric data. Post-hoc tests included the Bonferroni correction for

samples with homogenous variance and Dunnett's T3 for samples with heterogeneous variance. Statistically different means for non-normally distributed data were further analyzed by pairwise comparisons. A $P \leq 0.05$ was considered statistically significant.

Chapter IV: Results

Quantification of oxidation products in soybean oil: The effect of heat exposure on products of oxidation within soy oil relative to unheated oil was previously reported by our lab ³³. Results are presented in **Table 2**. As the duration of heat exposure increased, a corresponding significant increase in CDs was observed within 9hr-SO compared to all conditions. In contrast, the PV content increased slightly with 3hours of heating and then peaked at 6hours after which it steadily declined reaching a value below baseline (unheated oil) with 9hours of heat exposure. Secondary products (aldehydes) increased significantly between all oils whereas CT concentration within the 9hr-SO was no different from the 6hr-SO.

	Unheated SO	3h-SO	6h-SO	9h-SO
Peroxide Values	100%	108%	125% *^	77% ~
Conjugated Dienes	100%	596%	974%	1204% ~
Conjugated Trienes	100%	491%	600%	652% *^
Aldehyde Values	100%	1523% *	2971% *^	3925% ~

Table 2. Effect of heat duration on soy oil oxidation. Data are presented as percent change relative to unheated oil. P <0.05 is considered statistically significant; * significantly different than unheated oil; ^ significantly different than 3h-SO; ~ significantly different than each preceding stage; # significantly different than 6h-SO.

Cell Culture: Photo micrographs of untreated 3T3-L1 cells at the preadipocyte stage (3A) and mature fully differentiated 3T3-L1 cells (3B) are depicted in **Figure 3**.

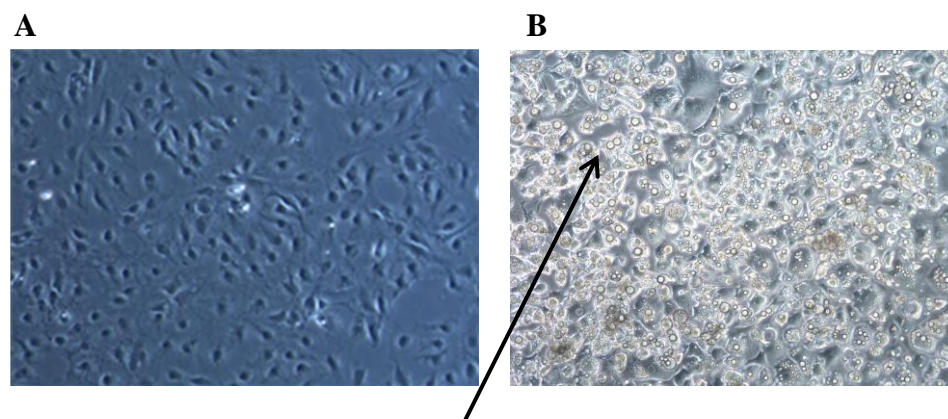


Figure 3. Differentiated untreated 3T3-L1 cells (A), mature adipocytes (B). Arrow indicates lipid droplet/TAG accumulation

PPAR γ transcription factor binding assay: 3T3-L1 cells were pretreated on day 1 with 0.01% unheated SO, 3hr-SO, 6hr-SO, or 9hr-SO for 24 hours. On days 6 and 15, the nuclear extracts were isolated and assayed to determine the influence of the 24 hour soy oil exposure on the ability of PPAR γ to bind to PPRE, relative to unheated SO (**Figure 4**). By day 6, 3T3-L1 cells treated with 6hr-SO demonstrated a noteworthy decrease in PPAR γ nuclear binding activity by -21%. A more modest decrease of -3% was observed with the 3hr-SO treated cells, while the 9hr-SO treated cells actually increased binding activity by 6%. By day 15, the mature 3T3-L1 cells pretreated with 3hr-SO demonstrated a -33% decrease in binding activity however the 6hr-SO and 9hr-SO treated cells increased binding activity by 21% and 6%, respectively.

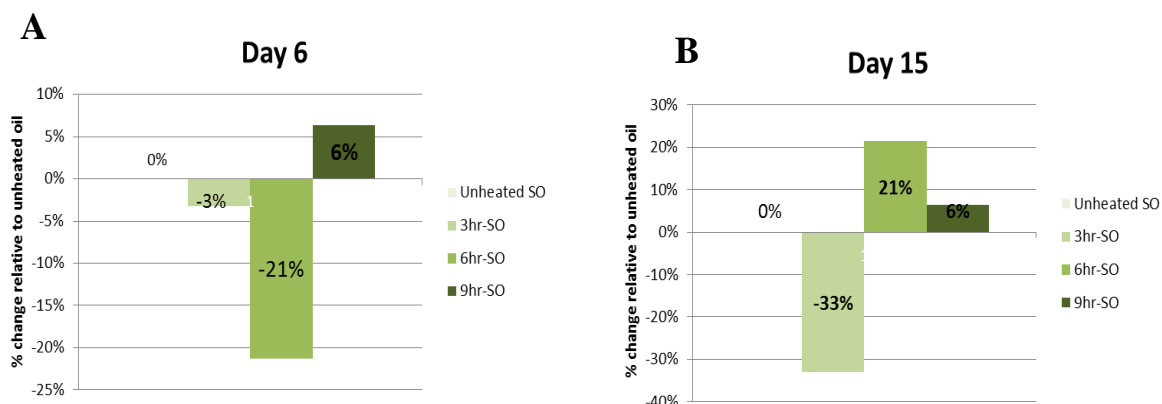


Figure 4. PPAR γ transcription factor binding in nuclear extracts from 3T3-L1 after a one time treatment with 0.01% unheated soy oil (SO), 3h heated (3h-SO), 6h heated (6h-SO) or 9h heated (9h-SO) on Day 6 (**A**) in partially differentiated cells and Day 15 (**B**) in Mature, fully differentiated cells. PPAR γ DNA activity is expressed as percent change relative to the unheated SO.

Gene Expression in 3T3-L1 cells after a one time treatment with oxidized soy oil:

RNA quality was confirmed by gel electrophoresis within 100% confluent cells, control cells, unheated SO, 3hr-SO, 6hr-SO, and 9hr-SO treated cells (**Figure 5**). The gene expression of SREBP-1c, PPAR γ , LPL, and GLUT-4 within 3T3-L1 cells pretreated for 24hours with unheated SO or oxidized SO was assessed at days 2, 6, 10, and 15 of the culture and differentiation process (**Figure 6**).

Upstream expression of SREBP-1c (Figure 6A): Looking at day 2, SREBP1c mRNA expression was reduced before the induction of differentiation within 3T3-L1 cells pretreated with heated soy oil compared to unheated SO. Occurring early in the differentiation process on day 6, SREBP-1c expression is further decreased within cells pretreated with 6hr-SO ($p=0.039$) whereas cells treated with the 9hr-SO demonstrated a significant reduction in expression by day 10.

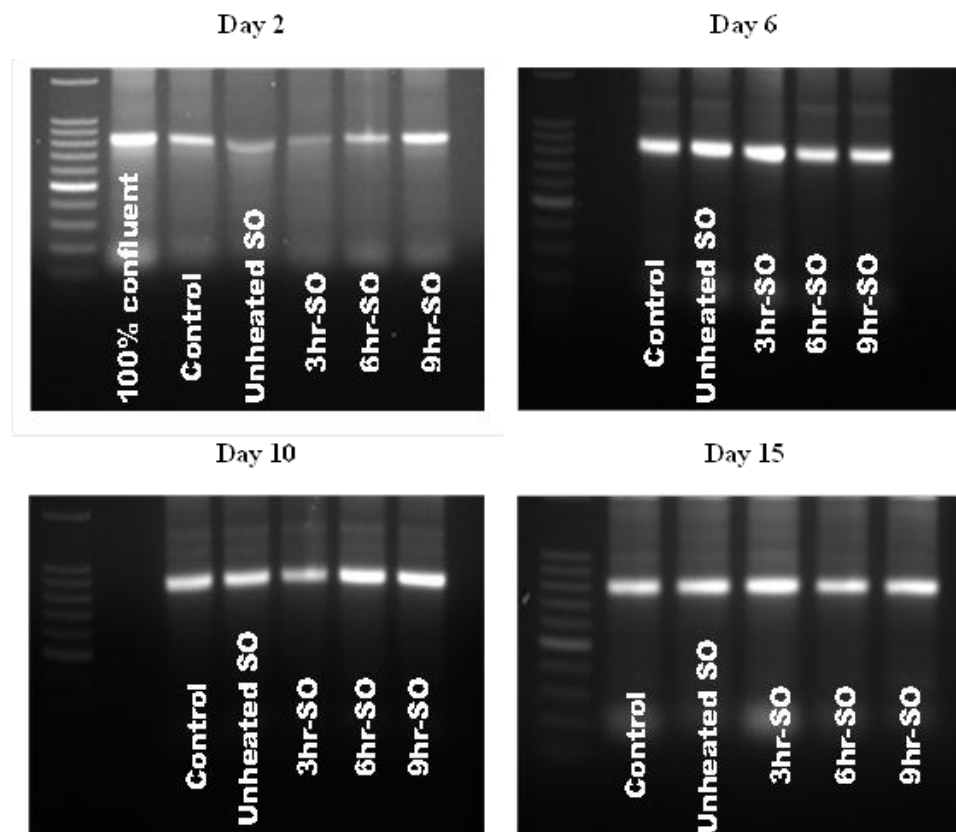


Figure 5. The presence and quality of isolated RNA were confirmed by the presence of solid, intact bands using gel electrophoresis.

PPAR γ Expression (Figure 6B): Cells pretreated with 6hr-SO demonstrated a significant decrease in PPAR γ mRNA by day 6 compared to all the treatment groups ($P < 0.05$ for all). After day 6, cells treated with the 6hr-SO then demonstrated an significant increase in PPAR γ expression at day 10 compared to 9hr-SO ($p = 0.002$) and at day 15 in comparison to 3hr-SO ($p = 0.013$). Cells treated with the 9hr-SO demonstrated a reduction in PPAR γ expression which reached significance by day 10 compared to unheated SO ($p = 0.027$).

Downstream Expression of LPL and GLUT-4 (Figures 6C and 6D): The changes in PPAR γ expression are reflected in the gene transcription of downstream LPL and

GLUT-4. On day 2, or 24 hours post-treatment, the expression of LPL and GLUT-4 is significantly greater within cells treated with heated oil compared to unheated SO (P<0.05 for all). Both LPL and GLUT-4 decrease significantly early in the differentiation process on day 6 within 6hr-SO treated cells. After day 6, LPL expression gradually increases overtime in 6hr-SO treated cells mimicking the increase in the expression of PPAR γ .

Influence of unheated SO and oxidized SO on adipogenic gene expression in 3T3-L1 cells

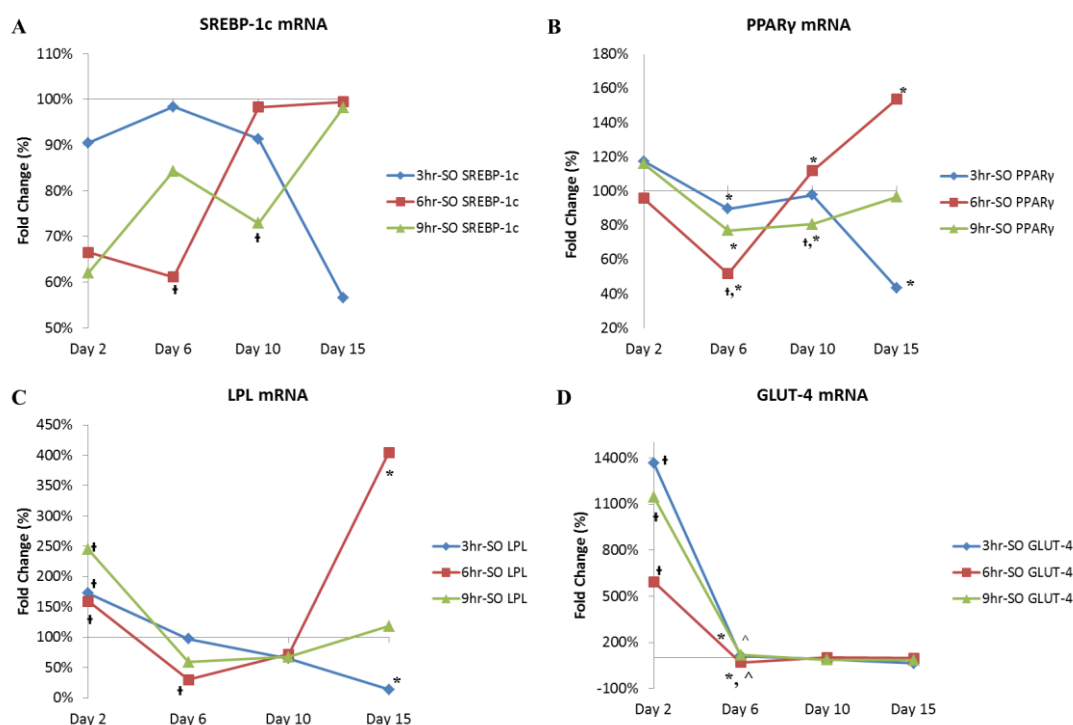


Figure 6. SREBP1c (6A), PPAR γ (6B), LPL (6C) and GLUT-4 (6D) mRNA expression within 3T3-L1 pretreated for 24 hours before the induction of differentiation with unheated SO, 3hr-SO, 6hr-SO, or 9hr-SO. Results represent the percent fold change relative to unheated oil for each given day. The data are summarized from triplicates (means \pm SEM). Mean differences in mRNA expression between groups was determined by one-way ANOVA followed by Bonferroni pair-wise comparisons for normally distributed data or by nonparametric tests where appropriate. For each day, heated oil treatments with significant differences in gene expression as compared to unheated SO are represented by (\dagger). Like symbols (* and ^) represent significant differences in gene expression between heated oils on a given day.

Influence of soy oil treatment on the PPAR γ levels: The result of soybean oil exposure on PPAR γ protein levels are presented as preliminary data as triplicates are needed in order to conclude any statistically noteworthy effects. PPAR γ protein levels within cells exposed to 6hr-SO were much lower before the induction of differentiation. By day 6, there was a decrease in the levels of PPAR γ protein within all cells exposed to heated oil compared to cells treated with unheated SO. PPAR γ levels remained suppressed in the 6hr-SO and 9hr-SO treated cells by day 10. By the end of differentiation on day 15, all cells exposed to heated oils demonstrated an increase in PPAR γ protein levels (**Figure 7**).

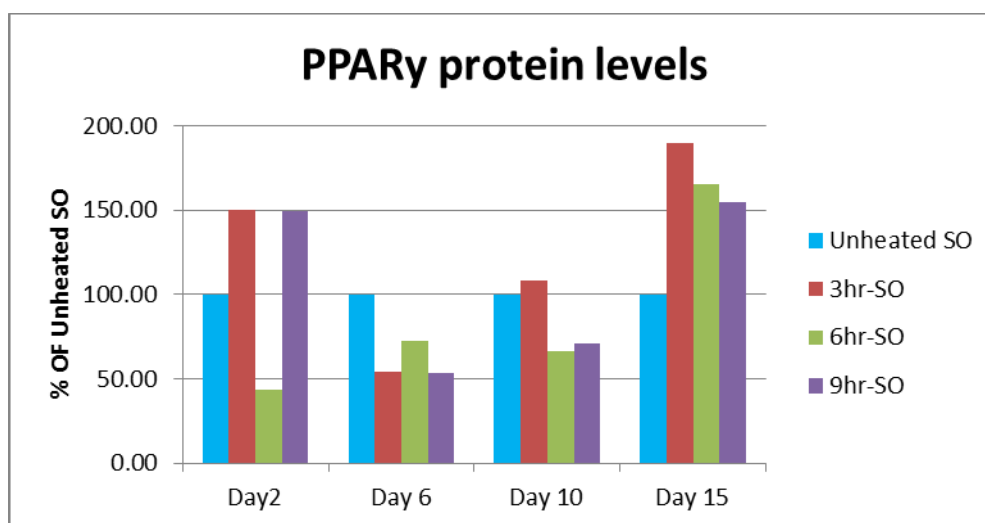


Figure 7. PPAR γ protein levels determined by Western blot. Protein levels within 3T3-L1 cells treated with oxidized SO are expressed in relation to the cells treated with unheated SO at Day 2, 6, 10 and 15.

Chapter V: Discussion and Conclusion

Oxidized lipids have been shown to reduce fat mass¹³ and TAG³⁴ accumulation however the mechanisms behind these alterations remain unclear¹³. The present study investigated the influence of oxidized/heated soybean oil on markers of TAG accumulation and PPAR γ activity within 3T3-L1 cells. Results indicate that the extent to which soy oil is heated or oxidized greatly influences PPAR γ gene expression, nuclear binding of PPAR γ to its response element, and downstream adipogenic gene expression. Results of the present study are in coincide with the previously reported alterations in TAG accumulation within 3T3-L1 cells treated with soy oil which was oxidized for ≥ 6 hours³⁴.

Cells exposed to a one-time treatment with 0.01% 6hr-SO exhibited the greatest reduction in PPAR γ DNA binding, along with significant reductions in the expression of PPAR γ target genes by day 6. Interestingly, this observation was preceded by a reduction in SREBP-1c and PPAR γ mRNA, suggesting that the decline in PPAR γ nuclear binding activity was a result of a reduction in PPAR γ expression occurring early in the differentiation process. This reduction might be related to the coinciding decrease in SREBP-1c expression and a >50% reduction in PPAR γ protein levels compared to all other oils. Suppressing SREBP-1c mRNA expression is linked to a reduction in TAG accumulation in vitro^{17,35}. C/EBP α , another transcription factor that was not measured in this study, regulates lipid metabolism by enhancing insulin sensitivity and reinforcing PPAR γ expression¹⁰. Previous studies have indicated that the absence of C/EBP α did not

alter adipocyte differentiation, TAG accumulation,¹⁰ or the expression of PPAR γ and GLUT-4³⁶ whereas PPAR γ deficient cells are unable to differentiate with C/EBP α alone¹⁰. In our study, the reduction by day 6 in the expression of downstream GLUT-4 within 6hr-SO treated cells demonstrates and suggests a specific relationship between PPAR γ and GLUT-4 gene expression, which is important early in the differentiation process.

Lipoprotein lipase is an additional PPAR γ target gene that was significantly altered in cells exposed to highly oxidized soybean oil. By day 6, LPL mRNA expression was down-regulated significantly within the 6hr-SO treated cells and modestly within 9hr-SO treated cells as compared to unheated SO and 3hr-SO. By day 10, mRNA expression levels remained lower compared to unheated SO; however, by day 15, the expression levels significantly increased in the 6hr-SO treated cells to almost 4-times that of the unheated SO. The sudden spike in LPL gene expression in the late phase of differentiation is likely related to the increase in SREBP-1c and PPAR γ mRNA expression by day 15; however, the reasoning and molecular mechanisms for this delayed response are unclear. It is possible that the potent oxidation products within the 6hr-SO are acting in such a way that expression is delayed or slightly inhibited. Therefore the lack of TAG accumulation we previously observed in mature adipocytes may indicate that cells exposed to more highly oxidized soy oil require a longer time for differentiation based on the late expression of PPAR γ and downstream LPL and GLUT-4. Conversely, cells which were pretreated with the mildly oxidized 3h-SO demonstrated a decrease in the expression of PPAR γ and downstream LPL at the end of the differentiation process. Studies have shown that the expression of PPAR γ is not necessary within mature

adipocytes as they have already accumulated TAGs ²⁴. Taken together, it appears that the gene expression within cells treated with unheated or mildly heated SO is reflective of TAG accumulating adipocytes.

Interactions between dietary lipids and PPAR γ activity are not restricted to adipose tissue alone. Gayet *et al.* found that diet-induced obesity within dogs on a hypercaloric, high-fat diet significantly reduced PPAR γ , LPL and GLUT-4 gene expression within visceral adipose tissue and skeletal muscle ³⁷. The observed simultaneous decrease in GLUT-4 expression within skeletal muscle suggests PPAR γ influence on gene expression expands beyond adipose tissue alone. Thus, interactions between PPAR γ and dietary oxidized lipids may not be limited to TG accumulation in adipose tissue alone, but have a greater influence on insulin-sensitive tissues. Moreover, Lapsys *et al.* found a significant correlation between PPAR γ and LPL expression within human skeletal muscle ³⁸. Considering the relationship and influence of PPAR γ on GLUT-4 and LPL expression in vivo, the alterations in gene expression observed in the presence of oxidized soybean oil are likely influencing muscle tissue as well as TG accumulation. A relationship between a higher intake of oxidized lipids and lower markers of adiposity has been shown in an epidemiological study using NHANES data ³⁹. This study found a positive relationship between oxidized lipid intake and plasma glucose levels ³⁹. Highly oxidized dietary soy oil has been shown to not only result in lesser weight gain but also lesser fat mass accumulation and TAG accumulation within rats ¹³. Of importance, this effect of highly oxidized dietary oil was accompanied by a decrease in insulin sensitivity and apparent hyperglycemia. If adipose tissue is unable to accumulate TAGs then it is likely TAGs may be accumulating in non-adipose tissues.

Thus it appears moderate- to highly- oxidized lipids may be altering more than simply fat accumulation and this observation may relate to the development of diabetes or insulin resistance.

PPAR γ activity may be influenced by several factors including upstream gene expression and/or the binding of agonistic/antagonistic ligands. The decrease in both SREBP-1c and PPAR γ mRNA expression suggests that highly oxidized soy oil is capable of influencing PPAR γ before PPAR γ ligand interactions. The ability of oxidized lipids to act as PPAR γ ligands remains of interest and these interactions may be occurring alongside changes in gene expression. Remarkably, the 6hr-SO contained the highest concentration of peroxides, primary products of oxidation, and also exhibited the greatest effects on PPAR γ activity and expression in the present study as well as the observed alterations in TAG accumulation in our previous study. On the other hand, the PV of the 9hr-SO was lower than any of the heated oils, including the unheated soy oil. The difference in PVs between the 6hr-SO and 9hr-SO may be highlighting a negative effect of peroxides on PPAR γ activity and adipogenic gene expression and, more importantly, may explain the differences in gene expression between the more highly oxidized oils. The relatively unchanged gene expression in the 9hr-SO treated cells conflicts with the previously observed lack of TAG accumulation within cells treated with the highly oxidized oil. Considering the results of the 6hr-SO treated cells and lack of TAG accumulation it is likely that a combination of factors, including oxidative stress, are influencing TAG accumulation. Of importance, both the 6hr-SO and 9hr-SO treated cells demonstrated a noteworthy increase in the nuclear binding of PPAR γ by the end of differentiation process. This finding again suggests a potential interaction on TAG

accumulation and PPAR γ activity independent of ligand interactions or it may be possible that secondary products of oxidation influence differentiation by modes other than PPAR γ . To our knowledge, primary oxidation products have been shown to act as ligands of PPAR γ whereas secondary products are not well noted to interact with PPAR γ . While the PV decreased after 6 hours of heating, we observed an increase in the concentration of CDs and secondary products as heating duration increased. Conjugated linoleic acid (CLA), which contains a conjugated diene structure, is responsible for decreasing TAG content within murine and human adipocytes, however the influence of CLA on adipogenic gene expression is conflicting and inconsistent⁴⁰. Possibly, the altered TAG accumulation within 9hr-SO treated cells is modulated by the presence CDs while the 6hr-SO seems to have a heightened effect, which is attributable to a combination of peroxides and CDs. CLA has been shown to be a weak PPAR γ ligand and its influence on adipogenesis is isomer specific. Brown *et al.* found *trans*-10, *cis*-12 but not the *cis*-9, *trans*-11 isomer of CLA to greatly decrease TAG accumulation and the expression of GLUT-4 and LPL⁴⁰. With the exception of aldehydes, the products of oxidation within the 3hr-SO were not significantly different compared to the products of oxidation within the unheated SO. Mice fed a low-fat diet with mildly oxidized soy oil heated for 3-hours were found to gain less weight compared to mice fed unheated SO however mice fed the heated SO had an increase in fat pad mass indicating a general prepotency to accumulate TAGs with mildly oxidized SO consumption⁵. In our study, cells pretreated with unheated SO or 3hr-SO did not demonstrate a significant difference in the expression of any of the genes of interest by day 6, which appears to be a critical time-point in the differentiation process. This may explain our previous observations where cells exposed

to mildly oxidized SO were able to differentiate while TAG accumulation was abolished with exposure to highly oxidized SO.

A limitation to consider when interpreting the results of this study concerns the fact that we only measured some of secondary oxidation products (aldehydes and CTs). For a more accurate interpretation, we should account for total secondary products by using a more thorough technique such as mass spectrophotometry. Additionally, our study assessed the influence of oxidized soybean oil, naturally rich in omega-6 fatty acids. The type of oil and its unique fatty acid composition exhibit considerable variability in the ability to influence PPAR γ activity and TAG accumulation.

Conclusion The early reduction in the gene expression of PPAR γ , LPL and GLUT 4 by day 6 in cells pretreated with 6hr-SO correlates the most with the lack of TG accumulation by day 15. Highly oxidized soybean oil appears to alter adipocyte differentiation by modulating PPAR γ expression early in the differentiation process resulting in a decrease in PPAR γ protein levels and subsequent decrease in nuclear binding and downstream gene expression. The mechanisms behind these observations require further analysis in order to determine if there is a relationship between oxidized lipids, PPAR γ activity, and glucose intolerance. Further experiments in murine models should facilitate this understanding and our lab is currently conducting such trials.

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